ACETYLCHOLINE RECEPTOR: CHANNEL-OPENING KINETICS EVALUATED BY RAPID CHEMICAL KINETIC AND SINGLE-CHANNEL CURRENT MEASUREMENTS

JAYANT B. UDGAONKAR AND GEORGE P. HESS
Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

ABSTRACT A combination of rapid chemical kinetic (quench-flow) and single-channel current measurements was used to evaluate kinetic parameters governing the opening of acetylcholine-receptor channels in the electric organ (electroplax) of *Electrophorus electricus*. Chemical kinetic measurements made on membrane vesicles, prepared from the *E. electricus* electroplax, using carbamoylcholine (200 μM–20 mM) at 12°C, pH 7.0, and in the absence of a transmembrane voltage, yielded values for K_1 (dissociation constant for receptor activation), Φ (channel closing equilibrium constant), $\bar{J}$ (specific reaction rate for ion flux), and α_{max} (maximum inactivation rate constant) of 1 mM, 3.4, 4×10^7 $M^{-1} s^{-1}$, and 12 s^{-1}, respectively. The single-channel current recordings were made with cells also from the *E. electricus* electroplax, at the same temperature and pH as the chemical kinetic measurements, using carbamoylcholine (50 μM–2 mM), acetylcholine (500 nM), or suberyldicholine (20 nM). Single-channel current measurements indicated the presence of a single, unique open-channel state of the *E. electricus* receptor, in concurrence with previous, less extensive measurements. The rate constant for channel closing (k_c) obtained from the mean open time of the receptor channel is 1,100 s^{-1} for carbamoylcholine, 1,200 s^{-1} for acetylcholine, and 360 s^{-1} for suberyldicholine at zero membrane potential; and it decreases *e*-fold for an 80 mV decrease in transmembrane voltage in each case. The decrease in mean open times of the receptor channel that is associated with increasing the carbamoylcholine concentration is interpreted to be due to carbamoylcholine binding to the regulatory (inhibitory) site on the receptor. An analysis of data obtained with carbamoylcholine showed that the closed times within a burst of channel activity fit a two-exponential distribution, with a concentration-independent time constant considered to be the time constant for carbamoylcholine to dissociate from the regulatory site, and a carbamoylcholine concentration-dependent, but voltage-independent, time constant interpreted to represent the rate constant for channel opening (k_0). An analysis of the mean closed time data on the basis of the minimum model gives values for K_1 and k_0 of 0.6 mM and 440 s^{-1}, respectively, with carbamoylcholine as the activating ligand. The values obtained for K_1, Φ ($= k_c/k_0$), $\bar{J}$, and α from the single-channel current measurements using electroplax are in good agreement with the values obtained from the chemical kinetic measurements using receptor-rich vesicles prepared from the same cells.

These results confirm the assumed basic agreement between two entirely different methodologies and underlie the strategy of using the two techniques to obtain complementary information in time and ligand-concentration regions where only one or the other technique can be used. This agreement between results allows estimates to be made of the k_0 values, for both acetylcholine and suberyldicholine, from the Φ values obtained from the chemical kinetic measurements and the k_c values obtained in single-channel current measurements.

INTRODUCTION

The acetylcholine receptor at the junction between nerve and muscle cells of vertebrates and between nerve and electroplax cells in the electric organ of electric fish responds to the binding of acetylcholine or other specific ligands by forming transmembrane ion-conducting channels. An amazing amount of progress has recently been made in investigations of the structure and function of this protein (reviewed in Stroud and Finer-Moore, 1985; Hess et al., 1987). Here we report on the evaluation of kinetic parameters governing the opening of acetylcholine-receptor channels in the electric organ (electroplax) of *Electrophorus electricus*. A combination of rapid chemical kinetic (quench-flow) and single-channel current measurements was used.

Studies of receptor-controlled currents have established that the channel opening and closing processes are rapid, occurring in the submillisecond to millisecond time domain. Electrophysiological methods, especially those

Dr. Udgaonkar's present address is Department of Biochemistry, Stanford University, Stanford, CA 94305.

Correspondence and offprint requests should be addressed to Dr. Hess.

using the single-channel recording technique (Neher and Sakmann, 1976*a*), have been used extensively to study the channel-opening kinetics of the acetylcholine receptor (reviewed in Adams, 1981; Sakmann and Neher, 1984). These studies have been hampered by difficulties in using a wide enough range of concentration of the activating ligand, necessary to arrive at a plausible model to interpret the data. At high ligand concentrations, a rapid (millisecond time region) inactivation process (desensitization), which was first discovered in rapid chemical kinetic measurements with receptor-containing vesicles prepared from the electroplax of *E. electricus* (Hess et al., 1978, 1979; Aoshima et al., 1981; and reviewed in Udgaonkar and Hess, 1986), abolishes the measurable signals and results in a protein with altered ligand-binding properties (reviewed in Changeux et al., 1984). For investigations of the kinetic properties of the receptor in the physiologically relevant time region before receptor desensitization, we used fast reaction techniques (Hess et al., 1979; Cash and Hess, 1980; Aoshima et al., 1981; Cash et al., 1981; Pasquale et al., 1983; Takeyasu et al., 1983, 1986; Shiono et al., 1984). Extensive investigations of the receptor in the electroplax of electric fish led to a proposed minimum model (Hess et al., 1983; Pasquale et al., 1983) that accounts for activation, inactivation (desensitization), and voltage-dependent regulation of the receptor by acetylcholine, carbamoylcholine, and suberyldicholine. The basis on which the chemical kinetic measurements of the receptor in vesicles prepared from electroplax can be compared to single-channel current measurements on the same electroplax cells has been established (Hess et al., 1984), and, more recently, a method for exposing and cleaning the surface of electroplax to make the cells suitable for single-channel current measurements has been reported (Pasquale et al., 1986). The acetylcholine receptor from the electroplax of *E. electricus* is at present the only receptor that can be studied in both individual cells, using the single-channel current recording technique, and membrane vesicles prepared from the same cells, using rapid chemical kinetic techniques.

In the experiments reported here we used the acetylcholine receptor of the *E. electricus* electroplax because chemical kinetic measurements have indicated (reviewed in Hess et al., 1983; Udgaonkar and Hess, 1986) that this receptor is not completely inactivated, even in the presence of high concentrations of activating ligand in the minute time region (Aoshima et al., 1981; Aoshima, 1984). Hence, the receptor can be studied by the single-channel recording technique, using much higher ligand concentrations than are possible with receptors from, for example, *Torpedo californica* (Tank et al., 1983; Suarez-Isla et al., 1983) or frog (Sakmann et al., 1980). Carbamoylcholine was chosen as the activating ligand because chemical kinetic measurements (Cash and Hess, 1980; Cash et al., 1981) have shown that it has a lower affinity for the non-desensitized receptor than does acetylcholine, and thus much higher concentrations can be used to obtain concentration-dependent changes of the rate constants. This is important in measurements of the rate constants involved in the channel-opening process; the ability to use high (millimolar) concentrations of carbamoylcholine simplifies analysis of the kinetic measurements because it ensures that the bimolecular ligand-binding steps preceding the actual channel-opening conformational change are not rate limiting in the receptor activation process (see Table I, Eq. 6). We shall demonstrate that the elucidation of concentration-dependent steps is possible by using carbamoylcholine concentrations from 50 μM to 2 mM, and that the results of our single-channel measurements corroborate the results of rapid chemical kinetic measurements made at the same temperature. The agreement of results obtained by chemical kinetic measurements and by single channel current measurements is used to estimate the channel-opening rate constant when acetylcholine and suberyldicholine are the activating ligands.

MATERIALS AND TECHNIQUES

Rapid Chemical Kinetic (Quench-Flow) Measurements

The preparation of receptor-rich membrane vesicles from *E. electricus* electroplax has been described previously (Kasai and Changeux, 1971; Fu et al., 1977). All measurements were carried out using vesicles equilibrated with eel Ringer's solution (OER) (169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0).

The fast influx of radioactive tracer metal ions, measured before and during inactivation (desensitization), was followed in the millisecond to second time region by using quench-flow techniques modified for use with membrane vesicles (Hess et al., 1979; Cash and Hess, 1981). Linear and nonlinear least-squares programs were used to obtain an optimal fit of the data to the theory (Bevington, 1969).

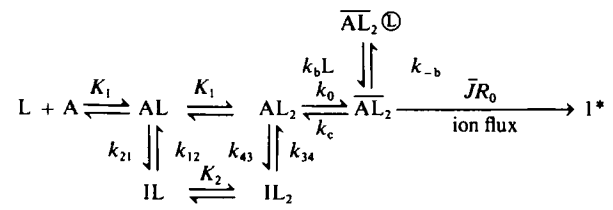
For experiments at 12°C, membrane vesicles, otherwise kept at 1°C, were warmed to 12°C an hour before the actual measurements. In each experiment, measurements with a saturating (1 mM) concentration of acetylcholine were performed to allow a comparison of the results obtained with different membrane preparations. The values obtained for J_A (the rate coefficient for ion transport before receptor desensitization) in each experiment were normalized to each other by using the acetylcholine control to correct for the variability in the membrane preparations. The value of J_A depends not only on the ligand concentration, but also on the number of receptors per liter internal volume of the vesicles, which varies somewhat for different vesicle preparations (Hess et al., 1981).

Single-Channel Current Measurements

The method to expose and clean *E. electricus* electroplax to make them suitable for single-channel current measurements has been described in detail (Pasquale et al., 1986). The electroplax were dissected immediately after killing the fish, and stored in OER at 4°C, for a maximum of 3 d. All single-channel current measurements were made at 12°C, on excised patches of cell membrane in the "inside-out" configuration (Hamill et al., 1981). The solution on either side of the membrane patch was always OER.

A commercially available patch clamp system (List L/M-EPC-7) for recording currents from single channels was used to make the measurements. Data were stored on FM analog tape using a Racal Store 4DS recorder at 7.5-kHz bandwidth. For analysis data were first passed through a low pass filter (model 3322; Krohn-Hite Corp., Avon, MA)

TABLE I
EQUATIONS USED FOR EVALUATION OF KINETIC CONSTANTS



$M_t/M_\infty = 1 - \exp[-J_A(1 - e^{-\alpha t})\alpha^{-1} + J_1 t]$	2‡
$J_A = \bar{J}R_0[L^2/(L^2 + L^2\Phi + 2K_1\Phi L + K_1^2\Phi)][K_R/(K_R + L)]$	3a§
$J_A = \bar{J}R_0[L^2/(L^2 + L^2\Phi + 2K_1\Phi L + K_1^2\Phi)]$	3b
$(\bar{J}R_0/J_A - 1)^{1/2} = \Phi^{1/2} + \Phi^{1/2}K_1/[L]$	3c
$\ln[(M_\infty - M_t)(M_\infty)^{-1}]_T/\ln[(M_\infty - M_t)(M_\infty)^{-1}]_{T=0} = e^{-\alpha T}$	4‖
$1/\bar{\tau}_0 = k_c + k_b[L]$	5a¶
$1/\bar{\tau}_0 = k_c$	5b
$1/\bar{\tau}_c = k_0[L/(L + K_1)]^2$	6a**
$(\bar{\tau}_c)^{1/2} = k_0^{-1/2}[(L + K_1)/L]$	6b
$1/\bar{\tau}_b = k_{-b}$	7‡‡
$P_0 = \bar{\tau}_0/(\bar{\tau}_0 + \bar{\tau}_c) = L^2/(L^2 + L^2\Phi + 2K_1\Phi L + K_1^2\Phi)$	8§§
$1/\bar{\tau}_A = \alpha$	9‖‖
$\bar{J} = NRT\gamma/F^2[M]$	10¶¶

*The mechanism relating ligand binding to acetylcholine receptor–controlled ion flux in *E. electricus* electroplax vesicles has been described in detail (Hess et al., 1983; Pasquale et al., 1983) and is depicted in Eq. 1. The regulatory binding site is considered to be present on all receptor conformations (Pasquale et al., 1983). However, for clarity, regulatory binding to only the open-channel state ($\overline{AL_2}$) is shown. The receptor can exist in an active closed (A), an active open ($\overline{A}$), and an inactive (I) (desensitized) state. A further desensitization process, which occurs in the hour time region (Aoshima et al., 1984), is not considered in the model. L represents ligand concentration. AL and AL_2, IL and IL_2 represent receptor species to which one or two (as indicated by the subscript) ligand molecules are bound. Ⓛ indicates that the ligand is bound to the regulatory site. For aesthetic reasons the addition of L to species AL and IL is omitted from the drawing of the mechanism. The rate constants for the formation of the inactive (desensitized) states (IL and IL_2) from AL and AL_2 are designated by k_{12}, k_{21}, and k_{34}, k_{43}, respectively. $\bar{J}$ is the specific reaction rate for the ion translocation process and is independent of both the activating ligand used and the receptor concentration (Hess et al., 1981). R_0 represents the moles of receptor per liter internal volume of the vesicles. k_c is the rate constant for channel closing and k_0 the rate constant for channel opening. k_b is the rate constant for ligand binding to the regulatory (inhibitory) site on the receptor and k_{-b} the rate constant for ligand dissociating from this site. K_1 and K_2 are the intrinsic dissociation constants of the complexes involving the active (A) and inactive (I) forms of the receptor, respectively. K_R is the dissociation constant of the complexes involving the regulatory site on the receptor, $\Phi = k_c/k_0$ is the channel-closing equilibrium constant.

‡M_t and M_∞ represent the tracer ion content of vesicles at time t and at equilibrium, respectively. J_A is the rate constant for ion flux before the onset of inactivation, α is the rate constant for receptor inactivation, and J_1 is the rate constant for ion flux after equilibration between the A and I states has been reached.

§The minimum mechanism yields this expression for J_A (Cash and Hess, 1980; Pasquale et al., 1983). When $L \ll K_R$, binding to the regulatory site is insignificant, and Eq. 3a simplifies to Eq. 3b. Eq. 3b can be rearranged to Eq. 3c (Hess et al., 1981).

‖α is evaluated using Eq. 4 as described by Aoshima et al. (1981). The symbol T represents the duration of preincubation with ligand and the subscript t the time during which ion flux is allowed to proceed after preincubation.

¶When $\overline{AL_2}$ can close either by the normal closing process (associated with the first-order rate constant k_c) or as a result of ligand binding to the regulatory site (associated with the second-order rate constant k_b), $\bar{\tau}_0$, the mean lifetime of the open channel is given by Eq. 5a. When binding to the regulatory site is insignificant ($L \ll K_R$) Eq. 5a reduces to Eq. 5b.

**Under conditions when a rapid preequilibrium exists between the ligand and the closed-channel form (A, AL, AL_2), i.e., at high [L] when the ligand-binding steps will be much faster than the channel-opening process, the mean lifetime of these closed-channel states, $\bar{\tau}_c$ is given by Eq. 6a. Eq. 6b is a transformation of Eq. 6a.

‡‡The mean lifetime of the blocked state, $\bar{\tau}_b$, is given by Eq. 7 when the only path from the blocked state $\overline{AL_2}$ L is to $\overline{AL_2}$.

§§In Eq. 8, P_0 is the conditional probability that the receptor channel is open when the receptor is in the non-desensitized state. It is equal to $[\overline{AL_2}]_0$ where $[\overline{AL_2}]_0 = \overline{AL_2}/(A + AL + AL_2 + \overline{AL_2})$, the fraction of receptor molecules in the open-channel form. Note that this expression for P_0 does not incorporate receptor inhibition. The ligand dependency of P_0 is the same as that of J_A in the absence of receptor inhibition (Eq. 3b). At high concentrations of ligand, when inhibition by activating ligand occurs, the ligand dependency of P_0 will be the same as that of J_A in Eq. 3a.

‖‖When, in single-channel current measurements, the periods of high channel activity (bursts) are much shorter in duration than the periods during which channel activity is not observed (interburst periods), the mean lifetime of the active periods (bursts), $\bar{\tau}_A$, is given by Eq. 9 (Hess et al., 1984).

¶¶$\bar{J}$ is related to the single-channel conductance by Eq. 10 (Hess et al., 1984). R, T, F, and N represent the gas constant, absolute temperature, Faraday constant, and Avogadro's number, respectively, and [M] is the molar concentration of the inorganic ions being transported.

with the cutoff frequency (−3 dB point) adjusted such that the average baseline deviation was 8 to 10 times less than the unit amplitude of the single-channel current. After filtering, the data were digitized at a sampling frequency (10 or 20 kHz) at least five times the cutoff frequency of the lowpass filter, using a PDP 11/23 minicomputer and stored on a hard disc. The data were then transferred to a Prime 750 computer (Material Science Center, Cornell University) for actual analysis. An automated analysis program, based on and modified from the program (IPROC) developed by Sachs et al. (1982), was used to detect events (the detection threshold was half the unit amplitude of events) and to prepare event amplitude and duration histograms. Typically a channel-open-time histogram, a histogram of the duration of periods of high channel activity (burst duration histogram), an event amplitude histogram, and three channel-closed-time histograms, each on a different time scale, were prepared. Nonlinear least-squares programs were used to perform fits on the histogram distributions. Exponential fits to open- and closed-time duration histograms were Poisson weighted. The goodness of the fit (reduced χ^2 tests) was tested.

Valid Events. In the construction of open time histograms, overlapping open events were ignored. No correction was made for the consequent bias against long openings. At high concentrations of channel-activating ligand, the recording is seen to consist of periods of activity (bursts) separated from one another by longer periods of no channel activity, as observed earlier with frog muscle receptors (Sakmann et al., 1980). The bursts and the intervals between bursts are taken to represent the lifetime of the receptor in its active and desensitized states respectively (Sakmann et al., 1980). In the construction of burst duration histograms, only bursts that met certain criteria were considered (Neher, 1983). Any period of activity was considered a valid burst and accepted for analysis

only if (*a*) there were no overlapping open events, and (*b*) it was preceded and followed by silent periods with durations of at least three times the mean closed time measured within the burst. In the construction of closed-time histograms, only gaps between events in valid bursts were accepted for analysis.

RESULTS

Single-channel currents recorded from acetylcholine receptor-channels of an electroplax dissected from the organ of Sachs of *E. electricus* are shown in Fig. 1. It has been shown previously (Pasquale et al., 1986) that these receptors have a single-channel conductance and a mean open-channel lifetime that are indistinguishable from those of the receptors from the main organ of *E. electricus*. The single-channel conductance, γ, at 12°C is ~50 pS, as measured using 50 to 500 μM carbamoylcholine, 20 nM suberyldicholine, or 0.1 μM acetylcholine as the activating ligand, in agreement with previous results (Pasquale et al., 1986). No subconductance states were detected.

The dependence of J_A, the rate coefficient for ion flux before the onset of receptor desensitization, on carbamoylcholine concentration, as measured in quench-flow experiments using vesicles prepared from the main organ of *E. electricus*, at 12°C and zero transmembrane voltage, is shown in Fig. 2 *a*. Data obtained at 1°C (Cash and Hess, 1980) are also presented for comparison. The determination of J_A by use of Eq. 2 in Table I and the derivation of the equation have been described in detail (Hess et al., 1983). Ion flux is seen to be faster at the higher temperature. At the higher concentrations of carbamoylcholine used, there is no observed reduction is J_A, as predicted by Eq. 3a (Table I) for the general model (Pasquale et al., 1983) and as observed in the case of suberyldicholine and acetylcholine (Pasquale et al., 1983; Takeyasu et al., 1983, 1986). This is a consequence of the fact that, in the range of carbamoylcholine concentrations studied, the value of the dissociation constant of the inhibitory site, K_R, of the receptor (Takeyasu et al., 1983, 1986; Shiono et al., 1984) is much higher than the carbamoylcholine concentrations used and Eq. 3a reduces to Eq. 3b (Table I). In Fig. 2 *b* the dependence of the desensitization rate coefficient, α, on carbamoylcholine concentration at 12°C is compared with the data obtained at 1°C. The evaluation of α has been described in detail (Aoshima et al., 1981). The data in Fig. 2 *a* were replotted according to Eq. 3c (Table I) in 2 *c*.

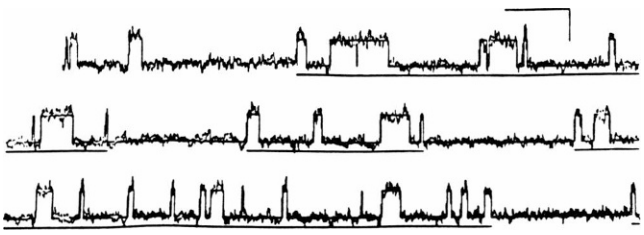


FIGURE 1 An example of a recording of single-channel currents through acetylcholine receptors of *E. electricus* electroplax using 500 μM carbamoylcholine at −100 mV, 12°C. Valid bursts have been identified by lines drawn above them. The period of activity at the end of the recording (lowest line, far right) was not accepted as a valid burst because of the presence of overlapping channel openings. The horizontal and vertical calibration bars represent 20 ms and 5 pA, respectively. Idealized computer-generated data are drawn through the actual data.

Single-channel recordings on acetylcholine receptor-channels from *E. electricus* electroplax were made using carbamoylcholine concentrations from 50 μM to 2 mM, or 20 nM suberyldicholine, or 0.1 μM acetylcholine. Typically measurements were made at five transmembrane voltages (V_m) in the range −80 to −160 mV for each carbamoylcholine concentration. An example of the channel open-time distribution is shown in Fig. 3 *a*. The distribution of channel-open times, at all concentrations of activating ligand and at all values of the transmembrane voltage, V_m, fitted a single exponential, showing that a unique open-channel state conformation existed for the acetylcholine receptor molecule. This is in concurrence with the minimum mechanism (Eq. 1, Table I) and in agreement with our previous report (Pasquale et al., 1986). In Fig. 3, *b* and *c* it is shown that the closed times within a burst follow a two-exponential distribution. This is true for carbamoylcholine concentrations in the range 100 μM to 2 mM. The faster time constant, designated $\bar{\tau}_b$, is independent of carbamoylcholine concentration and is taken to represent the lifetime of the complex formed by the ligand and the regulatory (inhibitory) site while the receptor is in the open-channel form (Eq. 1, Table I) (Pasquale et al., 1983). The time resolution of our measurements did not permit a sufficiently accurate determination of the voltage dependence of $\bar{\tau}_b$. The slower time constant, $\bar{\tau}_c$, was found to be dependent on carbamoylcholine concentration but independent of the transmembrane voltage. $\bar{\tau}_c$ is taken to represent the lifetime of the closed form of the channel within a burst at high ligand concentrations, when a rapid preequilibrium exists between the ligand and the closed-channel forms (A, AL, AL_2) of the receptor (Hess et al., 1984).

Fig. 3 *d* shows that the histogram of the duration of burst of channel activity follows a single exponential distribution with a time constant $\bar{\tau}_A$. $\bar{\tau}_A$, taken to represent the mean lifetime of the active state of the receptor before desensitization (Sakmann et al., 1980), is directly related to the rate coefficient for receptor desensitization, α, at the ligand concentrations used (Hess et al., 1984) (Eq. 9, Table I). $\bar{\tau}_A$ was found to decrease with an increasingly negative transmembrane voltage (data not shown). The voltage dependence of $\bar{\tau}_0$ (the mean lifetime of the open channel) and $\bar{\tau}_c$ (the mean liftime of the closed form of the channel) is shown in Fig. 4 *a*. The data obtained using 50 μM carbamoylcholine show that $\bar{\tau}_0$ is exponentially dependent on the transmembrane voltage; it increases *e*-fold for a decrease in transmembrane voltage of 80 mV. $\bar{\tau}_0$ reflects not only the channel-closing rate constant, k_c, but also the rate constant, k_b, for the binding of ligand to the regulatory site. This binding step depends on the ligand concentration, L (see Eq. 5a). At low ligand concentrations, when the

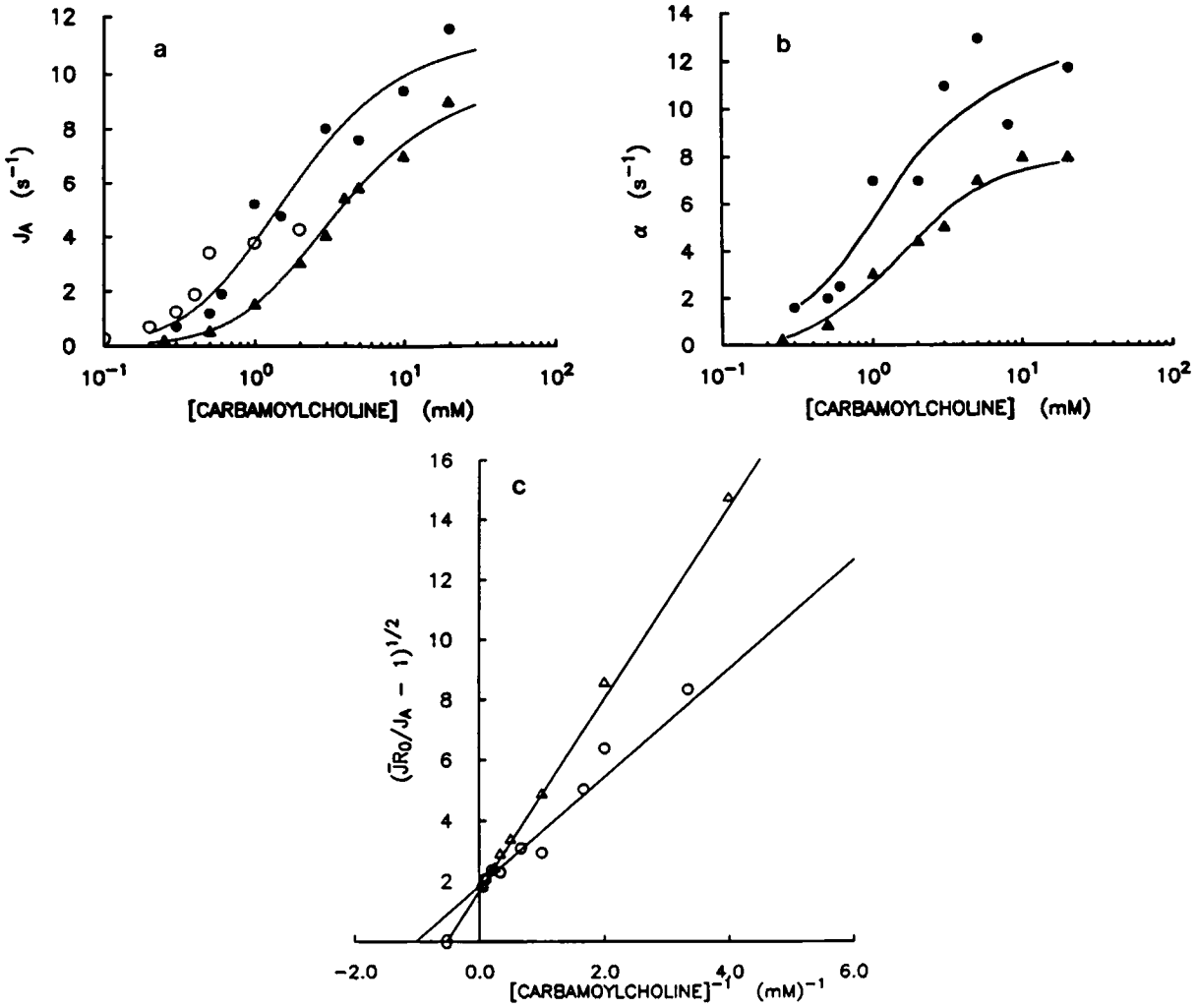


FIGURE 2 Effect of temperature on the ion translocation process mediated by the acetylcholine receptor in membrane vesicles prepared from *E. electricus* electroplax. ▲, 1°C; ●, 12°C. (*a*) Effect on the dependence of the influx rate constant, J_A, on carbamoylcholine concentration. 1 vol of membrane vesicles equilibrated with eel Ringer's solution (OER) was mixed with an equal volume of OER containing $^{86}Rb^+$ (33 μCi/ml final concentration) and various concentrations of carbamoylcholine. After various periods of incubation, t, the reaction was quenched by the addition of 5 vol of *d*-tubocurarine (25 mM). J_A values were calculated from the influx curves obtained by using Eq. 2 (Table I). The solid lines were calculated using Eq. 3b (Table I) and values for K_1, Φ, and $\bar{J}R_0$ of 1.9 mM, 2.8, and 37 s^{-1} at 1°C, and 1 mM, 3.4, and 50 s^{-1} at 12°C (see Fig. 2 *c*). The open circles represent P_0 values, determined from single-channel current measurements, calculated using Eq. 8 (Table I), and the values obtained in the absence of a transmembrane voltage for $\bar{\tau}_c$ (the mean channel-closed time) and for $\bar{\tau}_0$ (the mean channel-open time) shown in Fig. 4. To allow comparison of the two measurements, the P_0 value obtained at 500 μM carbamoylcholine was normalized to the J_A value obtained at the same carbamoylcholine concentration (see Eqs. 3b and 8 in Table I). (*b*) Effect on the dependence of the inactivation rate constant, α, on carbamoylcholine concentration. 1 vol of membrane vesicles equilibrated with OER was mixed with an equal volume of OER containing different concentrations of carbamoylcholine. After various times of exposure to carbamoylcholine, T, the activity was assayed in the second incubation of 1.5 s with $^{86}Rb^+$ (33 μCi/ml final concentration) as described in detail (Aoshima et al., 1981). The carbamoylcholine concentration was kept constant during incubation and influx. The values for α were calculated using Eq. 4 (Table I). (*c*) A linear plot of the relationship between J_A and carbamoylcholine concentration. Data from *a* were replotted using Eq. 3c (Table I). A linear least-squares fit to the data (*solid line*) was used to obtain the values for K_1, Φ, and $\bar{J}R_0$, which were then used to draw the solid lines in *a*. The average error in the values for the kinetic parameters was ±20%.

regulatory site is not occupied, Eq. 5a (Table I) reduces to Eq. 5b. The mean open times of the channel, $\bar{\tau}_0$, obtained at different transmembrane voltages and at a carbamoylcholine concentration of 50 μM, cannot be distinguished experimentally from those obtained using 100 μM carbamoylcholine and thus the former concentration is sufficiently low for Eq. 5b to be used to relate $\bar{\tau}_0$ to k_c, the channel-closing rate constant. Hence, the value of k_c in the absence of a transmembrane voltage, obtained from extrapolation of the line fitted through the data obtained using 50 μM carbamoylcholine (Fig. 4 *a*), is 1,100 s^{-1}. At concentrations above 200 μM carbamoylcholine, $\bar{\tau}_0$ decreases linearly with carbamoylcholine concentration (data not shown), in agreement with Eq. 5a (Table I).

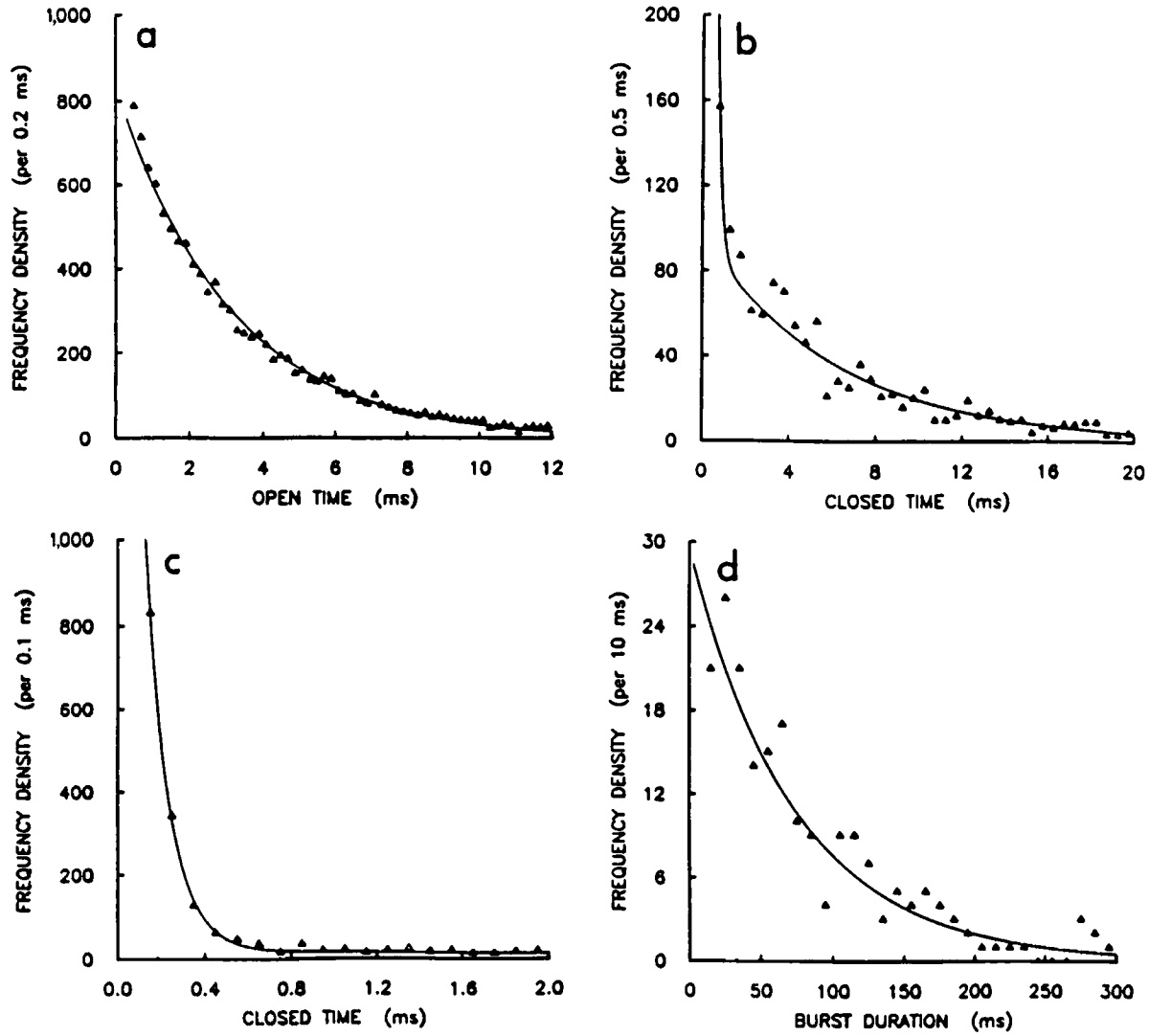


FIGURE 3. Statistical analysis of single-channel current data obtained with carbamoylcholine at 12°C, $V_m = -160$ mV on acetylcholine receptors from *E. electricus* electroplax. (*a*) Open time distribution. The histogram was fitted with a single exponential. The total number of detected events was 13,526. $\bar{\tau}_0 = 3.1$ ms. (*b* and *c*) Closed time distribution displayed on two different time scales. The histogram in *b* contains all events within valid bursts and was fitted by an unconstrained double exponential. The amplitude and time constant of the second slower component was then fixed and the faster component was fitted at higher resolution in *c*. The total number of events analyzed was 3,120. $\bar{\tau}_b = 0.11$ ms, $\bar{\tau}_c = 6.18$ ms. (*d*) Burst duration distribution. The histogram of valid burst durations was fitted by a single exponential. In this case the minimum closed time before and after a burst and separating the burst from other groups of events was 30 ms. The total number of bursts analyzed was 281. $\bar{\tau}_A = 73.8$ ms.

Channel-open-time data obtained with 500 μM carbamoylcholine, represented by solid squares, are shown in the same figure for comparison.

Also shown in Fig. 4 *a* (*solid circles, dotted line*) are the mean lifetime values of the closed form of the channel, $\bar{\tau}_c$, measured using 500 μM carbamoylcholine at five membrane potentials. $\bar{\tau}_c$ is observed to be independent of the transmembrane voltage. A mean closed-time of 6.3 ms is calculated (as the average of the values at the five different transmembrane voltages) for $\bar{\tau}_c$ at this concentration of carbamoylcholine. Fig. 4 *b* illustrates the sigmoidal dependence of the reciprocal mean channel-closed time, $\bar{\tau}_c$ on the carbamoylcholine concentration. The solid line in Fig. 4 *b* was drawn according to Eq. 6a (Table I) using values for K_1, the dissociation constant for carbamoylcholine binding to the site of the receptor that controls channel opening, and for k_0, the channel opening rate constant, which were obtained from the linear plot of the same data in Fig. 4 *c*. The ordinate intercept and the slope of the straight line in Fig. 4 *c* give K_1 and k_0 according to Eq. 6b.

The value obtained for $\bar{\tau}_0$ in the absence of a transmembrane potential and the voltage-independent value obtained for $\bar{\tau}_c$ were used to evaluate P_0, the conditional probability that the receptor channel is open, providing that the receptor is in the non-desensitized (active) state (Eq. 8, Table I). The open circles in Fig. 2 *a* represent the

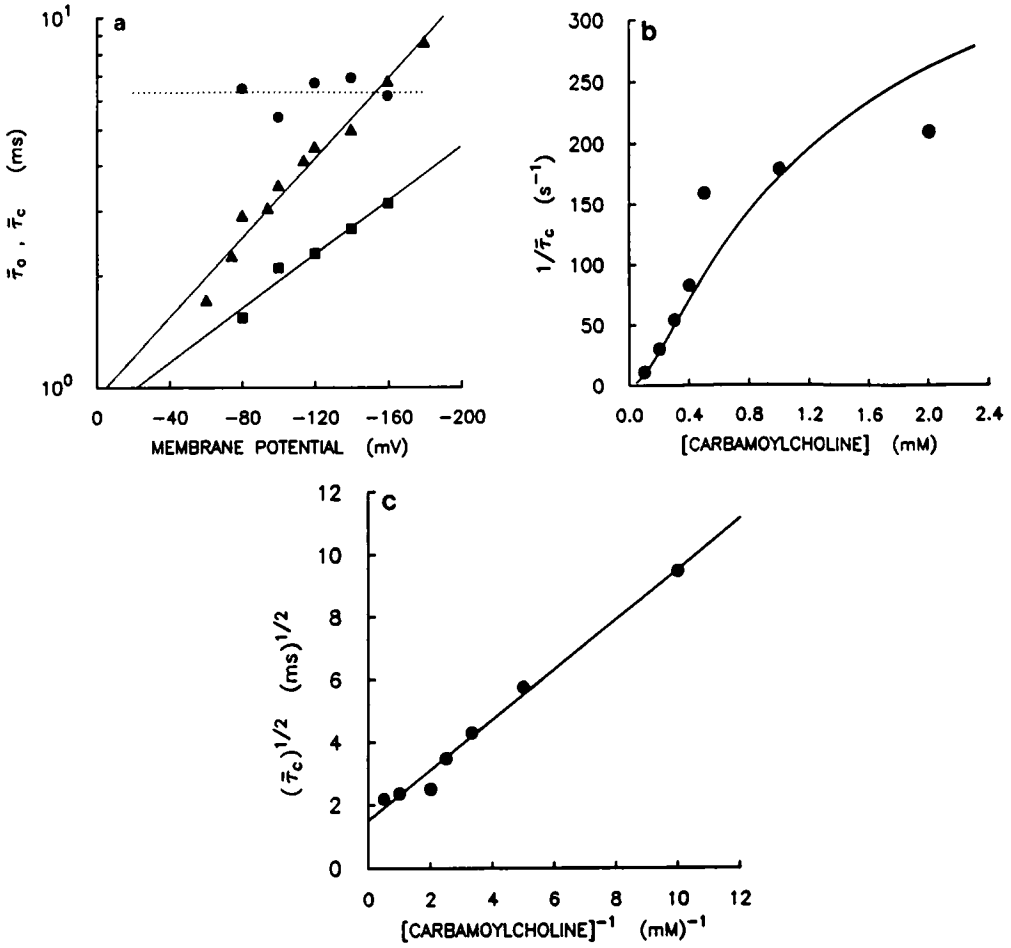


FIGURE 4 Carbamoylcholine concentration dependence and voltage dependence of acetylcholine receptor channel open and closed times measured from single-channel current data obtained with *E. electricus* electroplax at 12°C. (*a*) Voltage dependencies. ▲, mean open times, 50 μM carbamoylcholine; ■, mean open times, 500 μM carbamoylcholine; ●, mean closed times, 500 μM carbamoylcholine. The mean open time (τ_0) at each voltage was obtained from data such as that shown in Fig. 3 *a*. The straight line through the mean open times data for 50 μM carbamoylcholine shows that the mean open time is exponentially dependent on transmembrane voltage; it increases *e*-fold for a decrease in transmembrane voltage of 80 mV. Extrapolation of the fitted line to 0 mV yields a value for $\bar{\tau}_0$ of 0.9 ms at zero transmembrane voltage. The mean closed time ($\bar{\tau}_c$) at each voltage was obtained from the slower component of the two-exponential fit of closed times within a burst (Fig. 3 *b*). No observable dependence of $\bar{\tau}_c$ on transmembrane voltage was seen. The dotted line indicates the average mean closed time (6.3 ms) obtained as the average of the mean closed times at the five different transmembrane voltages. (*b*) Dependence of the mean closed time on carbamoylcholine concentration. The reciprocal of the average mean closed time at each carbamoylcholine concentration (see *a*) was plotted against carbamoylcholine concentration. The solid line was drawn according to Eq. 6a (Table I) using values for K_I and k_0 of 0.6 mM and 440 s^{-1} obtained in *c*. (*c*) The data in *b* were replotted according to Eq. 6b (Table I). A linear least-squares fit through the data yielded values for K_I and Φ calculated from the slope and intercept of the straight line.

P_0 values for seven carbamoylcholine concentrations at 12°C; they have been normalized to the J_A values at 12°C (Fig. 2 legend). As expected from theory, the effects of ligand concentration on J_A and P_0 are the same.

Mean open times at 12°C were also measured using very low suberyldicholine (20 nM) and acetylcholine (500 nM) concentrations. As in the case of 50 μM carbamoylcholine, these concentrations were low enough to prevent the ligand from binding to the regulatory (inhibitory) site of the receptor and to permit Eq. 5b to be used to relate the mean open-times to the channel-closing rate constants (k_c). k_c was evaluated from the dependence of $\bar{\tau}_0$ on the transmembrane voltage; for acetylcholine k_c = 1,200 s^{-1}, and for suberyldicholine k_c = 360 s^{-1}, at zero membrane potential. The former decreased *e*-fold for a decrease in transmembrane voltage of 76 mV; the latter decreased *e*-fold for a decrease in transmembrane voltage by 78 mV (actual data not shown).

DISCUSSION

In Table II, the values of kinetic parameters obtained using the single-channel current measurements are compared with those obtained using the chemical kinetic measurements. The interpretation of data from the rapid chemical kinetic measurements, in which the properties of a large population of receptors is measured, has a completely

TABLE II
COMPARISON OF KINETIC PARAMETERS EVALUATED FROM SINGLE-CHANNEL CURRENT MEASUREMENTS TO THOSE OBTAINED IN CHEMICAL KINETIC MEASUREMENTS IN PRESENCE OF CARBAMOYLCHOLINE, IN OER, pH 7.0

	Chemical kinetic measurements (12°C)	Single-channel current measurements (12°C)
$\bar{J}$ (Specific rate constant for ion flux)	$4 \times 10^7\ M^{-1}\ s^{-1}$	$4 \times 10^7\ M^{-1}\ s^{-1}$*
K_I (Dissociation constant for receptor complex controlling channel opening)	1 mM	0.6 mM
$\Phi = k_c/k_0$ (Channel-closing equilibrium constant)	3.4	2.5‡
α (Inactivation rate coefficient)	$12\ s^{-1}$	$20\ s^{-1}$§

*The value obtained by use of Eq. 10 (Table I), with $\gamma = 50$ pS at 12°C.
‡The value obtained using a value for $k_c = 1{,}100\ s^{-1}$ at $V_m = 0$ mV and for k_0 of $440\ s^{-1}$ (Fig. 4).
§The value obtained from data in Fig. 3 *d* using Eq. 9, Table I.

different basis from the interpretation of data from single-channel current measurements, in which the properties of a single receptor molecule are measured. The good correspondence between the values for the intrinsic rate constants governing acetylcholine receptor function obtained from these two completely different types of measurement is consistent with the minimum model (Eq. 1, Table I).

The verification of the value for the channel-closing equilibrium constant (Φ), obtained from chemical kinetic measurements, is especially noteworthy (Table II). In Table III are listed the values for the channel-closing rate constant, k_c, obtained with acetylcholine and suberyldicholine, together with the corresponding values for Φ, which have been determined previously from chemical kinetic measurements. Because $\Phi = k_c/k_0$, it is possible to estimate the value for the channel-opening rate constant, k_0, for both acetylcholine and suberyldicholine (Table III).

EFFECT OF TEMPERATURE ON THE INTRINSIC CONSTANTS GOVERNING ACETYLCHOLINE RECEPTOR FUNCTION

Though the rapid chemical kinetic measurements were made at 12°C to compare them to the single-channel current measurements made at the same temperature, we can also compare them to similar, previous measurements made at 1°C (Cash and Hess, 1980; Fig. 2 and accompanying legend). $\bar{J}$, the specific reaction rate for ion flux (Hess et al., 1981), was evaluated using Eq. 3c (Table I). In this equation $\bar{J}$ represents the specific reaction rate of the receptor-controlled transmembrane ion flux and R_0 the moles of receptor per liter internal vesicle volume (Hess et al., 1981). It has been shown previously that $\bar{J}$ is directly related to the single-channel conductance, γ (Hess et al., 1984). When R_0 is determined, excellent agreement is obtained between $\bar{J}$, determined by chemical kinetic measurements with vesicles, and γ, determined from single-channel current measurements using the electroplax cells from which the vesicles were prepared (Hess et al., 1984). Because measurements of J_A at 12°C included determinations of J_A in the presence of 1 mM acetylcholine, the $\bar{J}R_0$ values as well as the single-channel conductance can be compared with the values previously obtained at 1°C. It is thus possible to calculate $\bar{J}R_0$ at 12°C from the γ measurements made at 12°C. The value for $\bar{J}R_0$ that gave the best

TABLE III
ESTIMATES FOR THE CHANNEL OPENING RATE CONSTANT (k_0) FOR THREE DIFFERENT LIGANDS FROM MEASUREMENTS IN OER, pH 7.0

Ligand	Channel-closing rate constant (k_c)*	Channel-closing equilibrium constant ($\Phi = k_c/k_0$)	Estimated channel-opening rate constant (k_0)
Carbamoylcholine	$1{,}100\ s^{-1}$	2.8‡ (3.4)§	440** s^{-1}
Suberyldicholine	$360\ s^{-1}$	1.0‖	$360\ s^{-1}$
Acetylcholine	$1{,}200\ s^{-1}$	1.5¶	$800\ s^{-1}$

SCCM, single channel current measurements using electroplax cells. KM, kinetic measurements using the quench flow technique and membrane vesicles prepared from electroplax cells.
*All values for k_c are for $V_m = 0$ mV and 12°C (SCCM).
‡Value obtained at 1°C (Cash and Hess, 1980) (KM).
§Value obtained at 12°C (Fig. 2) (KM).
‖Value obtained at 1°C (Pasquale et al., 1983) (KM).
¶Value obtained at 1°C (Cash et al., 1981) (KM).
**Value actually obtained (Fig. 4) (SCCM).

fit to the data at 12°C was 50 s^{-1}; the value at 1°C has been shown to be 37 s^{-1} (Hess et al., 1981). The abscissa intercept provided a value for K_1, while the ordinate intercept yielded a value for $\Phi^{1/2}$. Values for these constants at 12°C are summarized in Table II. The error estimates for the values of these constants are from 20 to 30%. $\bar{J}$ is increased by a factor of 1.5 by a 10°C rise in temperature. The single-channel conductance, γ, increased by the same factor for a similar rise in temperature (Pasquale et al., 1986), as required by the relationship between $\bar{J}$ and γ (Eq. 10, Table I). Φ, the channel-closing equilibrium constant, is not significantly altered by a 10°C rise in temperature. That the channel-closing rate constant (k_c) is doubled by a 10°C rise in temperature (Pasquale et al., 1986) indicates that the channel-opening rate constant (k_o) has a similar temperature dependency as $k_c \cdot K_1$, the affinity of the receptor for ligand, is nearly doubled by a 10°C rise in temperature. α, the rate constant for receptor inactivation, evaluated from the values of $\bar{\tau}_A$ (Eq. 9, Table I) (Hess et al., 1984), is increased by a factor of 1.5 by a 10°C rise in temperature, over the entire range of carbamoylcholine concentrations studied. Measurements of $\bar{\tau}_A$ with acetylcholine as the activating ligand (unpublished observations) indicate a similar temperature-dependent increase in the values of α. The evaluations of $\bar{\tau}_A$ are in agreement with previous measurements using rapid chemical kinetic techniques (Takeyasu et al., 1983), which indicate that α is increased by a factor of ~1.5 as the transmembrane voltage changes from 0 to −48 mV. These results suggest that, under normal physiological conditions at the vertebrate synapse (37°C, −80 mV, and high concentrations of acetylcholine), inactivation (desensitization) of the receptor has a time constant of <25 ms. Desensitization may, therefore, be fast enough to play an important role in short-term information storage at the synapse (Hess et al., 1983; Changeux et al., 1984; Udgaonkar and Hess, 1986).

Channel-closing Rate Constant

The channel-closing rate constant (k_c) for the acetylcholine receptor of the *E. electricus* electroplax has been calculated from the decay rate constant of the nerve impulse–induced receptor-controlled current in the postsynaptic membrane (Sheridan and Lester, 1975, 1977) to be 1,200 s^{-1} at 15°C, and has been reported to decrease *e*-fold for a decrease in membrane potential of 86 mV. These values are in good agreement with the values for k_c and the voltage dependency of k_c we have obtained from single-channel current measurements at 12°C, with acetylcholine as the activating ligand, which are reported here. The mean open times measured with acetylcholine as the activating ligand are similar in magnitude to the values reported for acetylcholine receptors in the postsynaptic membrane of frog muscle cells (Sakmann et al., 1980), and for reconstituted *T. californica* receptors (Suarez-Isla et al., 1983; Tank et al., 1983). The value obtained for k_c with suberyldicholine as the activating ligand is 2 to 2.5 times less than the value obtained with either acetylcholine or carbamoylcholine as the channel-activating ligand, in agreement with previous work reported with reconstituted *Torpedo* receptor (Suarez-Isla et al., 1983). It is interesting that k_c has the same voltage dependence, regardless of whether the activating ligand is acetylcholine, carbamoylcholine, or suberyldicholine. The decrease in the mean open time ($\bar{\tau}_0$) at higher concentrations of channel-activating ligand, observed here with carbamoylcholine, was also observed in earlier single-channel measurements when suberyldicholine was used as the activating ligand with both BC_3H1 cells (Sine and Steinbach, 1984*b*) and *E. electricus* electroplax (Pasquale et al., 1986).

Channel-opening Rate Constant

The appearance of channel activity during short periods of time (bursts), which are observed in the single-channel current record when carbamoylcholine concentrations higher than 100 μM are used, was previously observed when acetylcholine concentrations higher than 2 μM were used in single-channel current measurements on frog acetylcholine receptor (Sakmann et al., 1980). That the mean interburst duration is longer, by more than an order of magnitude, than the mean closed time within a burst allows the assumption that only one receptor channel is opening and closing during a burst. The receptor is considered to be in an active state during a burst and in an inactive (desensitized) state during the interburst interval (Sakmann et al., 1980). Burst durations, reflecting a first-order rate coefficient for desensitization (Hess et al., 1979), are expected to follow a single exponential distribution (desensitization is a first-order process), as is observed (Fig. 3 *d*). The mean lifetime of the burst, $\bar{\tau}_A$, is a measure of the desensitization rate (Sakmann et al., 1980) and, at the ligand concentrations used, the desensitization rate coefficient, α, can be directly evaluated from $\bar{\tau}_A$ (Eq. 9, Table I) (Hess et al., 1984). The values of α obtained from chemical kinetic measurements and from single-channel current measurements are compared in Table II. The higher value for α obtained from the single-channel current measurements is probably a reflection of the fact that it was evaluated at a membrane potential of −160 mV, whereas the value from chemical kinetic measurements was obtained at zero transmembrane voltage. It has been shown previously (Takeyasu et al., 1983) that decreasing the transmembrane voltage increases the value for α. Because the receptor is in the active state during a burst, and because the carbamoylcholine concentrations used are high enough to assume that at these carbamoylcholine concentrations a preequilibrium exists between the closed active states, we used Eq. 6a (Table I) to obtain the channel-opening rate constant, k_0, and the ligand dissociation constant, K_1, from the ligand-dependency of the mean closed-time within a burst, $\bar{\tau}_c$. The voltage independence of k_0, which we observe (Fig. 4 *a*), was suggested previously

in noise analysis and voltage-jump relaxation studies (Neher and Sakmann, 1975).

The determination of the channel-opening rate constant, on the basis of our measurements using both the chemical kinetic and single-channel current measuring techniques (Table III), will now be compared with the results obtained in other studies. The ligand concentration-dependence of mean closed times ($\bar{\tau}_c$) was examined by Sakmann et al. (1980). In those experiments with frog muscles, the maximum acetylcholine concentration used was 50 μM and the maximum observed channel-opening rate constant ($1/\bar{\tau}_c$) was 500 s^{-1}. In previous measurements with frog muscle cells, in which the concentration dependence of the time constant obtained in noise analysis was measured, the highest concentration of acetylcholine used was 30 μM, and the estimate for k_0 was 2,000 s^{-1} (Sakmann and Adams, 1979).

When low concentrations (nanomolar) of activating ligand (acetylcholine, carbamoylcholine, or suberyldicholine) were used in single-channel current measurements with frog muscle cells, it was observed (Colquhoun and Sakmann, 1981, 1985; Sine and Steinbach, 1984*a*; Auerbach and Sachs, 1984) that an opening event was interrupted by very short (20–50 μs) gaps. A majority of these gaps were too fast to be resolved well enough to determine whether they represented transitions to a fully closed state or to a subconductance state. The mean duration of these gaps depended on the nature of the activating ligand but was independent of the concentration of the ligand. The frequency of the gaps was observed to be independent of the transmembrane voltage. In one of these reports it was postulated that these gaps represented the receptor undergoing very fast transitions to a closed state not involved in the actual activation of the receptor by ligand (Sine and Steinbach, 1984*b*). In the other reports (Colquhoun and Sakmann, 1981, 1985), despite the concentration-independence of the mean duration of these gaps, it was postulated that these very short gaps signified the channel-opening rate constant, k_0, and a value of 20,000 s^{-1} was obtained. The concentration-independence of k_0, at low acetylcholine concentrations observed in those studies, suggests that a process different from that described in the study described here was observed.

In voltage-jump relaxation studies of the acetylcholine receptor of *E. electricus* electroplax, a concentration-dependence of the relaxation time constant (which signifies the sum of the closing rate and the apparent opening rate of the receptor channel) was observed (Sheridan and Lester, 1975, 1977). At the highest concentration of acetylcholine used (100 μM), a relaxation rate constant of 800 s^{-1} at 15°C was obtained. The data are in good agreement with our results (see Table III).

Miniature end-plate currents, receptor-controlled currents that result from the random release of acetylcholine from the presynaptic nerve cells, have been observed in *E. electricus* electroplax by Lester et al. (1978). These currents rise to their peak within 400 μs at 20°–23°C; the corresponding rise times at the frog (Dwyer, 1981) and lizard (Land et al., 1980) muscle cell junctions are 250 and 100 μs, respectively, at the same temperature. The time taken for acetylcholine to diffuse across the synaptic cleft has been shown to contribute negligibly to the rise time of a miniature end-plate current (Dwyer, 1981). The rising phase of the miniature end-plate current is thus determined by the channel opening kinetics of the receptor, and the relaxation rate constants associated with the rise time will then be given by the sum of the closing (k_c) and apparent opening ($1/\bar{\tau}_c$) rate constants; the decay rate constant, on the other hand, is equal to k_c (Anderson and Stevens, 1973). We have determined in *E. electricus* at 12°C and −80 mV, that k_c for acetylcholine has a value of ~500 s^{-1} and, assuming a saturating concentration of acetylcholine at the synapse (Kuffler and Yoshikami, 1975), the apparent opening rate constant will be approximately k_0, which is estimated to be equal to ~800 s^{-1} (Table III). The rise time of the miniature end-plate current at 12°C is 2.3 times the rise time at 23°C (Dwyer, 1981). From the values of k_c and k_0 determined with the receptor in the electroplax cell of *E. electricus* at 12°C we calculate a rise time at 23°C and −80 mV of ~800 μs. This calculated rise time is in good agreement with the rise time determined at the *E. electricus* electroplax (Lester et al., 1978) and the estimated values for k_c and k_0 for acetylcholine reported here.

We thank Dr. Frederick Sachs, Department of Biophysical Sciences, State University of New York, Buffalo, for providing us with a copy of his program, IPROC, which we used for analyzing single-channel current measurements, Dr. Erwin Neher, Max Planck-Institute for Physical Chemistry, Göttingen, for allowing us to analyze some of our data there, thus allowing data analysis methods to be compared, and Dr. Elena Pasquale for preliminary measurements.

This work was submitted by J. Udgaonkar to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. We are grateful for the financial support provided by a grant (GM-04842) from the National Institutes of Health awarded to G. P. Hess.

Received for publication 27 July 1987.

REFERENCES

Adams, P. R. 1981. Acetylcholine receptor kinetics. *J. Membr. Biol.* 58:161–174.

Anderson, C. R., and C. F. Stevens. 1973. Voltage clamp analysis of acetylcholine-produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol. (Lond.).* 235:655–691.

Aoshima, H. 1984. A second, slower inactivation process in acetylcholine receptor-rich membrane vesicles prepared from *Electrophorus electricus*. *Arch. Biochem. Biophys.* 235:312–318.

Aoshima, H., D. J. Cash, and G. P. Hess. 1981. The mechanism of the inactivation (desensitization) of the acetylcholine receptor. Investigations by fast reaction techniques with membrane vesicles. *Biochemistry.* 20:3467–3474.

Auerbach, A., and F. Sachs. 1984. Flickering of a nicotinic ion channel to a subconductance state. *Biophys. J.* 45:187–198.

Bevington, P. 1969. Data Reduction and Error Analysis. McGraw-Hill Book Co., New York.

Cash, D. J., H. Aoshima, and G. P. Hess. 1981. Acetylcholine-induced cation translocation across cell membranes and inactivation of the acetylcholine receptor: chemical kinetic measurements in the millisecond time region. *Proc. Natl. Acad. Sci. USA.* 78:3318–3322.

Cash, D. J., and G. P. Hess. 1980. Molecular mechanism of acetylcholine-receptor-controlled ion translocation across cell membranes. *Proc. Natl. Acad. Sci. USA.* 77:842–846.

Cash, D. J., and G. P. Hess. 1981. Quenched flow technique with plasma membrane vesicles: acetylcholine-receptor-mediated transmembrane ion flux. *Anal. Biochem.* 112:39–51.

Changeux, J.-P., A. Devillers-Thiéry, and P. Chemouilli. 1984. Acetylcholine receptor: an allosteric protein. *Science (Wash. DC).* 225:1335–1345.

Colquhoun, D., and B. Sakmann. 1981. Fluctuations in the microsecond time range of the current through single acetylcholine receptor ion channels. *Nature (Lond.).* 294:464–466.

Colquhoun, D., and B. Sakmann. 1985. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *J. Physiol. (Lond.).* 369:501–557.

Dwyer, T. M. 1981. The rising phase of the miniature endplate current at the frog neuromuscular junction. *Biochim. Biophys. Acta.* 646:51–60.

Fu, J-j. L., D. B. Donner, D. E. Moore, and G. P. Hess. 1977. Allosteric interactions between the membrane-bound acetylcholine receptor and chemical mediator:equilibrium measurements. *Biochemistry.* 16:678–684.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann and F. J. Sigworth. 1981. Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch. Eur. J. Physiol.* 391:85–100.

Hess, G. P., H. Aoshima, D. J. Cash, and B. Lenchitz. 1981. The specific reaction rate of acetylcholine-receptor-controlled ion translocation: a comparison of measurements with membrane vesicles and with muscle cells. *Proc. Natl. Acad. Sci. USA.* 78:1361–1365.

Hess, G. P., D. J. Cash, and H. Aoshima. 1979. Acetylcholine receptor-controlled ion fluxes in membrane vesicles investigated by fast reaction techniques. *Nature (Lond.).* 282:329–331.

Hess, G. P., S. Lipkowitz, and G. E. Struve. 1978. Acetylcholine-receptor-mediated ion flux in electroplax membrane microsacs (vesicles): change in mechanism produced by asymmetrical distribution of sodium and potassium ions. *Proc. Natl. Acad. Sci. USA.* 75:1703–1707.

Hess, G. P., D. J. Cash, and H. Aoshima. 1983. Acetylcholine-receptor-controlled ion translocation. Chemical kinetic investigations of the mechanism. *Annu. Rev. Biophys. Bioeng.* 12:443–473.

Hess, G. P., H. A. Kolb, P. Läuger, E. Schoffeniels, and W. Schwarze. 1984. Acetylcholine receptor (from *E. electricus*): a comparison of single-channel current recordings and chemical kinetic measurements. *Proc. Natl. Acad. Sci. USA.* 81:5281–5285.

Hess, G. P., J. B. Udgaonkar, and W. L. Olbricht. 1987. Chemical kinetic measurements of transmembrane processes using rapid reaction techniques: acetylcholine receptor. *Annu. Rev. Biophys. Biophys. Chem.* 16:507–534.

Kasai, M., and J. -P. Changeux. 1971. *In vitro* excitation of purified membrane fragments by cholinergic agonists. I. Pharmacological properties of the excitable membrane fragments. *J. Membr. Biol.* 6:1–23.

Kuffler, S. W., and D. Yoshikami. 1975. The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. *J. Physiol. (Lond.).* 251:465–482.

Land, B. R., E. E. Salpeter, and M. M. Salpeter. 1980. Acetylcholine receptor site density affects the rising phase of miniature endplate currents. *Proc. Natl. Acad. Sci. USA.* 77:3736–3740.

Lester, H. A., D. D. Koblin, and R. E. Sheridan. 1978. Role of voltage-sensitive receptors in nicotinic transmission. *Biophys. J.* 21:181–194.

Neher, E. 1983. The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anesthetics. *J. Physiol. (Lond.).* 339:663–678.

Neher, E., and B. Sakmann. 1975. Voltage-dependence of drug-induced conductance in frog neuromuscular junction. *Proc. Natl. Acad. Sci. USA.* 72:2140–2144.

Neher, E., and B. Sakmann. 1976*a*. Single-channel currents recorded from membrane of denervated frog muscle fibers. *Nature (Lond.).* 260:799–802.

Neher, E., and B. Sakmann. 1976*b*. Noise analysis of drug-induced voltage clamp current in denervated frog muscle fibers. *J. Physiol. (Lond.).* 258:705–729.

Pasquale, E. B., K. Takeyasu, J. B. Udgaonkar, D. J. Cash, M. C. Severski, and G. P. Hess. 1983. Acetylcholine receptor: evidence for a regulatory binding site in investigations of suberyldicholine-induced transmembrane ion flux in *Electrophorus electricus* membrane vesicles. *Biochemistry.* 22:5967–5973.

Pasquale, E. B., J. B. Udgaonkar, and G. P. Hess. 1986. Single-channel current recordings of acetylcholine receptors in electroplax isolated from the *Electrophorus electricus* Main and Sachs' electric organs. *J. Membr. Biol.* 93:195–204.

Sachs, F., J. Neil, and N. Barkakati. 1982. The automated analysis of data from single ionic channels. *Pfluegers Arch. Eur. J. Physiol.* 395:331–340.

Sakmann, B., and P. R. Adams. 1979. Biophysical aspects of agonist action at frog endplate. *In* Advances in Pharmacology and Therapeutics. Vol. 1. J. Jacob, editor. Pergamon Press, Oxford.

Sakmann, B., and E. Neher. 1984. Patch clamp techniques for studying ionic channels in excitable membranes. *Annu. Rev. Physiol.* 46:455–472.

Sakmann, B., J. Patlack, and E. Neher. 1980. Single acetylcholine-activated channels show burst kinetics in presence of desensitizing concentrations of agonist. *Nature (Lond.).* 286:71–73.

Sheridan, R. E., and H. A. Lester. 1975. Relaxation measurements on the acetylcholine receptor. *Proc. Natl. Acad. Sci. USA.* 72:3496–3500.

Sheridan, R. E., and H. A. Lester. 1977. Rates and equilibria at the acetylcholine receptor of *Electrophorus* electroplax. A study of neurally evoked postsynaptic currents and of voltage-jump relaxations. *J. Gen. Physiol.* 70:187–219.

Shiono, S., K. Takeyasu, J. B. Udgaonkar, A. H. Delcour, N. Fujita, and G. P. Hess. 1984. Regulatory properties of acetylcholine receptor: evidence for two different inhibitory sites, one for acetylcholine and the other for a noncompetitive inhibitor of receptor function (procaine). *Biochemistry.* 23:6889–6893.

Sine, S. M., and J. H. Steinbach. 1984*a*. Activation of a nicotinic acetylcholine receptor. *Biophys. J.* 45:175–185.

Sine, S. M., and J. H. Steinbach. 1984*b*. Agonists block currents through acetylcholine receptor channels. *Biophys. J.* 46:277–284.

Stroud, R. M., and J. Finer-Moore. 1985. Acetylcholine receptor structure, function and evolution. *Annu. Rev. Cell Biol.* 1:369–401.

Suarez-Isla, B. A., K. Wan, J. Lindstrom, and M. Montal. 1983. Single-channel recordings from purified acetylcholine receptors reconstituted in bilayers formed at the tip of patch pipettes. *Biochemistry.* 22:2319–2324.

Takeyasu, K., S. Shiono, J. B. Udgaonkar, N. Fujita, and G. P. Hess. 1986. Acetylcholine receptor: characterization of the voltage-dependent regulatory (inhibitory) site for acetylcholine in membrane vesicles from *Torpedo californica* electroplax. *Biochemistry.* 25:1771–1776.

Takeyasu, K., J. B. Udgaonkar, and G. P. Hess. 1983. Acetylcholine receptor: evidence for a voltage-dependent regulatory site for acetylcholine. Chemical kinetic measurements in membrane vesicles using a voltage clamp. *Biochemistry.* 22:5973–5978.

Tank, D. W., R. L. Huganir, P. Greengard, and W. W. Webb. 1983. Patch-recorded single-channel currents of the purified and reconstituted *Torpedo* acetylcholine receptor. *Proc. Natl. Acad. Sci. USA.* 80:5129–5133.

Udgaonkar, J. B., and G. P. Hess. 1986. Acetylcholine receptor kinetics: chemical kinetics. *J. Membr. Biol.* 93:93–109.